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SOX11 identified by target gene evaluation of miRNAs differentially expressed in focal and non-focal brain tissue of therapy-resistant epilepsy patients

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally control the expression of their target genes via RNA interference. There is increasing evidence that expression of miRNAs is dysregulated in neuronal disorders, including epilepsy, a chronic neurological disorder characterized by spontaneous recurrent seizures. Mesial temporal lobe epilepsy (MTLE) is a common type of focal epilepsy in which disease-induced abnormalities of hippocampal neurogenesis in the subgranular zone as well as gliosis and neuronal cell loss in the cornu ammonis area are reported. We hypothesized that in MTLE altered miRNA-mediated regulation of target genes could be involved in hippocampal cell remodeling. A miRNA screen was performed in hippocampal focal and non-focal brain tissue samples obtained from the temporal neocortex (both $n = 8$) of MTLE patients. Out of 215 detected miRNAs, two were differentially expressed (hsa-miR-34c-5p: mean increase of 5.7 fold ($p = 0.014$), hsa-miR-212-3p: mean decrease of 76.9% ($p = 0.0014$)). After *in-silico* target gene analysis and filtering, reporter gene assays confirmed RNA interference for hsa-miR-34c-5p with 3′-UTR sequences of GABRA3, GRM7 and GABBR2 and for hsa-miR-212-3p with 3′-UTR sequences of SOX11, MECP2, ADCY1 and ABCG2. Reporter gene assays with mutated 3′-UTR sequences of the transcription factor SOX11 identified two different binding sites for hsa-miR-212-3p and its primary transcript partner hsa-miR-132-3p. Additionally, there was an inverse time-dependent expression of Sox11 and miR-212-3p as well as miR-132-3p in rat neonatal cortical neurons. Transfection of neurons with anti-miRs for miR-212-3p and miR-132-3p suggest that both miRNAs work synergistically to control Sox11 expression. Taken together, these results suggest that differential miRNA expression in neurons could contribute to an altered function of the transcription factor SOX11 and other genes in the setting of epilepsy, resulting not only in impaired neural differentiation, but also in imbalanced neuronal excitability and accelerated drug export.

Introduction

Epilepsy is a common chronic neurological disorder affecting more than 50 million people of all ages worldwide (Sisodiya, 2007). The neuronal hyperexcitabilty associated with seizures may be explained by an imbalance in excitatory and inhibitory signal transmission. However, the cellular and molecular mechanisms underlying increased susceptibility to recurrent seizures are not well understood. Mesial temporal lobe epilepsy (MTLE) is a common type of focal epilepsy often associated with pharmacoresistance and a histopathological finding of mesial temporal sclerosis (MTS) (Blumcke et al., 2007; Engel, 2001). Histopathological findings in a rat MTLE model and in human hippocampal specimens have demonstrated an increase in neurogenesis of dentate granule cells accompanied by a dispersed dentate granule cell layer and the ectopic location of granule cells in the hilus after acute seizures

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(Blumcke et al., 2001; Parent et al., 1997, 2006; Thom, 2004) which may contribute to excitatory circuitry in the hilus and CA3 region. In contrast, in the chronic phase of epilepsy with spontaneous recurrent seizures and signs of gliosis and neuronal cell loss in the cornu ammonis area, the number of neuronal stem cells (NSCs) seems not to change dramatically. However, the correct differentiation of NSC progenies into mature neurons appears to be impaired (Hattiangady and Shetty, 2008; Kuruba et al., 2009), potentially leading to areas of increased excitability that contribute to the development, maintenance and progression of epilepsy.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally fine-tune the expression of their target genes by interfering with the mRNA 3′-UTR region, resulting in mRNA degradation or inhibition of protein translation. miRNAs are involved in many important cellular processes including proliferation, differentiation, and maintenance of tissue specificity. The process of adult neurogenesis is associated with a miRNA-controlled sequence of altered expression of specific transcription factors and other proteins (Faigle and Song, 2013; Liu et al., 2010; Szulwach et al., 2010). Moreover, there is increasing evidence that expression of miRNAs is dysregulated in neuronal disorders, including Alzheimer's disease, depression, schizophrenia and bipolar disorder (Jin et al., 2013). Recent reports from screening studies in animal epilepsy models and from human specimens of MTLE patients show that miRNA expression is also altered in epilepsy (Dogini et al., 2013; Jimenez-Mateos and Henshall, 2013). Furthermore, epilepsy-dependent alterations in miRNA expression change between the acute post-seizure phase and the chronic, often intractable phase of spontaneous recurrent seizures (Bot et al., 2013; Song et al., 2011).

The current study is based on the hypothesis that hippocampal neuronal reorganization in the chronic phase of MTLE is associated with altered miRNA-mediated regulation of target genes predisposing the brain to recurrent epileptic seizures. After screening genome-wide miRNA expression in hippocampal focal and non-focal brain tissue from the temporal neocortex of MTLE patients who underwent temporal lobectomy, a reporter-gene assay was used to functionally investigate the interaction between dysregulated miRNAs and predicted target genes potentially involved in hippocampal cellular remodeling during epileptogenesis. Bioinformatic filtering for potential target genes included a hypothesis-free as well as a phenotype-guided approach. Candidate miRNAs and their functionally confirmed target genes identified in this study imply that differential hippocampal miRNA expression could contribute to altered function of several genes in the chronic stage of MTLE, resulting not only in impaired neural differentiation, but also in imbalanced neuronal excitability and accelerated drug export. Moreover, the results suggest that certain miRNAs act synergistically to control the expression of their target gene, emphasizing the complexity of miRNA networks in epilepsy.

Material and methods

Human tissue material

Eight hippocampal focal and eight non-focal brain tissue samples obtained from the temporal neocortex of 10 MTLE patients (six males and four females, mean age: 37.3 +/− 11.5 years), who underwent temporal lobectomy were screened for miRNA expression. The study protocol was approved by Committee on Human Research (CHR) at University of California, San Francisco, and informed consent was obtained from all participants. Demographic data, diagnosis, histopathological findings and history of pharmacotherapy are summarized in Table 1.

Paraformaldehyde-fixed, paraffin-embedded tissue slides of hippocampus and frontal cortex obtained from autopsies of eight individuals (four males and four females, mean age: 52.4 +/− 5.9 years) without neurological disorder were investigated for hsa-miR-212-3p expression. This investigation was approved by the ethics committee of the Medical Faculty of the University of Greifswald, Germany (BB43/09).

miRNA isolation and miRNA screen in human brain tissue of MTLE patients

After homogenization of frozen tissue specimens of MTLE patients on dry ice, total RNA was isolated using a mirVana™ PARIS™ Kit (Ambion by Life Technologies, Austin, TX, USA). Reverse transcription of 200 ng of total RNA per sample was performed using a TaqMan® MicroRNA Reverse Transcription Kit and miRNA specific RT primer pools (Megaplex™ Primer Pools, Human Pools Set v3.0, consisting of Megaplex™ RT Primers, Human Pool A v2.1 and Pool B v3.0, Applied Biosystems by Life Technologies, Foster City, CA, USA). Reverse transcription was followed by a subsequent pre-amplification step using a TaqMan® PreAmp Master Mix (Applied Biosystems) and pre-amplification primer pools (Megaplex™ PreAmp Primers, Human Pool A v2.1 and Pool B v3.0, Applied Biosystems). Resulting cDNAs were diluted 1:4 in 0.1X TE (1X TE-Buffer: Tris (10 mM), EDTA (1 mM), pH = 8.0, Ambion) and then loaded together with TaqMan® Universal Master Mix II, no UNG (Applied Biosystems) on low density arrays (final dilution 1:400, TaqMan® Array Human MicroRNA A + B Cards Set v3.0, Applied Biosystems) in a volume of 100 μl per port. Quantitative real-time PCR was run on an ABI prism 7900HT (Applied Biosystems). All described reactions were performed according to a standard protocol provided by the manufacturer. NormFinder software (Andersen et al., 2004) identified RNU48 as the most stably expressed housekeeping gene in both tissue types. miRNA expression was quantified as $2^{-\Delta Ct}$, where ΔCt is the difference in Ct values between the gene of interest and RNU48.

Principal component analysis and identification of differently expressed miRNAs

Only those miRNAs detected in all 16 brain tissue samples were included in the final comparison between focal hippocampal and non-focal temporal neocortical tissue. Visualization of miRNA expression data was done by heatmap and by Principal Component Analysis (PCA) in the statistical computing environment R/Bioconductor (Gentleman et al., 2004; R Development Core Team, 2010) (Fig. 1). Comparison between both tissue types was conducted by Mann–Whitney *U* test and multiple testing correction was performed using Benjamini and Hochberg False Discovery rate (FDR) (Benjamini and Hochberg, 1995) in R (Gentleman et al., 2004; R Development Core Team, 2010) with the HTqPCR package (Dvinge and Bertone, 2009). Differentially expressed miRNAs having a p-value ≤ 0.02 and a $\log_2$-transformed fold change ≥ 2.0 were considered relevant for further investigation.

Confirmation of selected candidate miRNA expression

Reverse transcription of 20 ng of total RNA, obtained from above mentioned frozen tissue specimens of MTLE patients, was conducted using respective RT primer pools and a TaqMan® MicroRNA Reverse Transcription Kit. Confirmation of array candidate miRNA expression was performed without a pre-amplification step using individual TaqMan® MicroRNA assays (hsa-miR-34c-5p: Assay ID 000428, hsa-miR-212-3p: Assay ID 000515, hsa-miR-221: Assay ID 000524, RNU48: Assay ID 001006) and TaqMan® Universal Master Mix II, no UNG (all reagents purchased from Applied Biosystems). Total RNA was isolated from paraformaldehyde-fixed, paraffin-embedded tissue slides of autopsies using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion). After reverse transcription and a subsequent pre-amplification step, expression of hsa-miR-212-3p and RNU48 was measured using respective TaqMan® MicroRNA assays. Both reactions, either with or without a pre-amplification step, were conducted according to a protocol provided by the manufacturer.

Table 1
Demographic data, diagnosis, pharmacotherapy and type of examined brain tissue of MTLE patients.

Patient	Gender	Age at surgery (years)	Age at onset of disease	Type of examined brain tissue	Diagnosis /Histopathology	Anticonvulsant	
						at surgery	prior
1	female	30	10 months	Hippocampus	Medically refractory epilepsy with temporal lobe focus; MTS confirmed	CBZ	VAP, TGB, PBT
2	female	46	2 years	Hippocampus	Medically refractory epilepsy with temporal lobe focus; MTS confirmed	ZNS, DVP	DVP, TGB
3	male	62	41 years	Temporal neocortex	Medically refractory epilepsy with temporal lobe focus; MTS confirmed	CBZ	DVP, ZNS, LEV, PHT, LTG, TPR, CLZ
4	female	23	5-6 years	Temporal neocortex	Medically refractory epilepsy with temporal lobe focus; signs of MTS	LTG, GBP	PHT, CBZ, PBT, DVP, ZNS, GBP
5	male	34	24 years	Hippocampus, temporal neocortex	Medically refractory epilepsy with temporal lobe focus; no signs of MTS	DVP, CBZ	PHT, GBP, CBZ, DVP
6	female	27	25 years	Hippocampus, temporal neocortex	Medically refractory epilepsy with temporal lobe focus; signs of MTS	LTG, OXC	LEV, PHT, unknown multiple other anticonvulsants
7	male	35	18 months	Hippocampus, temporal neocortex	Medically refractory epilepsy with temporal lobe focus, MTS confirmed	OXC, LEV, LZP	LTG, unknown multiple other anticonvulsants
8	male	51	42 years	Hippocampus, temporal neocortex	Medically refractory epilepsy with temporal lobe focus; MTS confirmed	DVP, CBZ	PHT, DVP, CBZ
9	male	36	9 months	Hippocampus, temporal neocortex	Medically refractory epilepsy with temporal lobe focus; MTS confirmed	CBZ, PGB, LZP	PBT VAP, LTG, LEV, LZP
10	male	29	early childhood	Hippocampus, temporal neocortex	Medically refractory epilepsy with temporal lobe focus; signs of MTS	DVP, OXC	unknown multiple other anticonvulsants

MTS: mesial temporal sclerosis, CBZ: carbamazepine, CLZ: clonazepam, DVP: divalproex, GBP: gabapentin, LTG: lamotrigine, LEV: levetiracetam, LZP: lorazepam, OXC: oxcarbazepine, PBT: phenobarbital, PHT: phenytoin, PGB: pregabaline, TGB: tiagabine, TPR: topiramate, VAP: valproic acid, ZNS: zonisamide.

In silico analysis of target genes of selected candidate miRNAs

Two approaches were conducted in parallel to extract genes predicted to be targeted by selected candidate miRNAs. One approach (hypothesis-guided) accounted for ATP-binding cassette (ABC) transporters known to be involved in drug resistance (ABCB1, ABCC1, ABCC2, ABCC4, ABCG2), a phenomenon often observed in the chronic stage of MTLE (Engel, 2001), as well as for genes related to GABAergic and glutamatergic signal transmission. ABC transporters were tested for predicted miRNA/mRNA interaction using four different prediction databases, namely TargetScan release 5.2 (Grimson et al., 2007), RNAHybrid (Rehmsmeier et al., 2004), PicTar (Krek et al., 2005) and miRanda (Betel et al., 2008). GABAergic and glutamatergic signal transmission-related target genes were extracted from gene lists generated using the GOmir software application (Roubelakis et al., 2009), which refers to those four different databases.

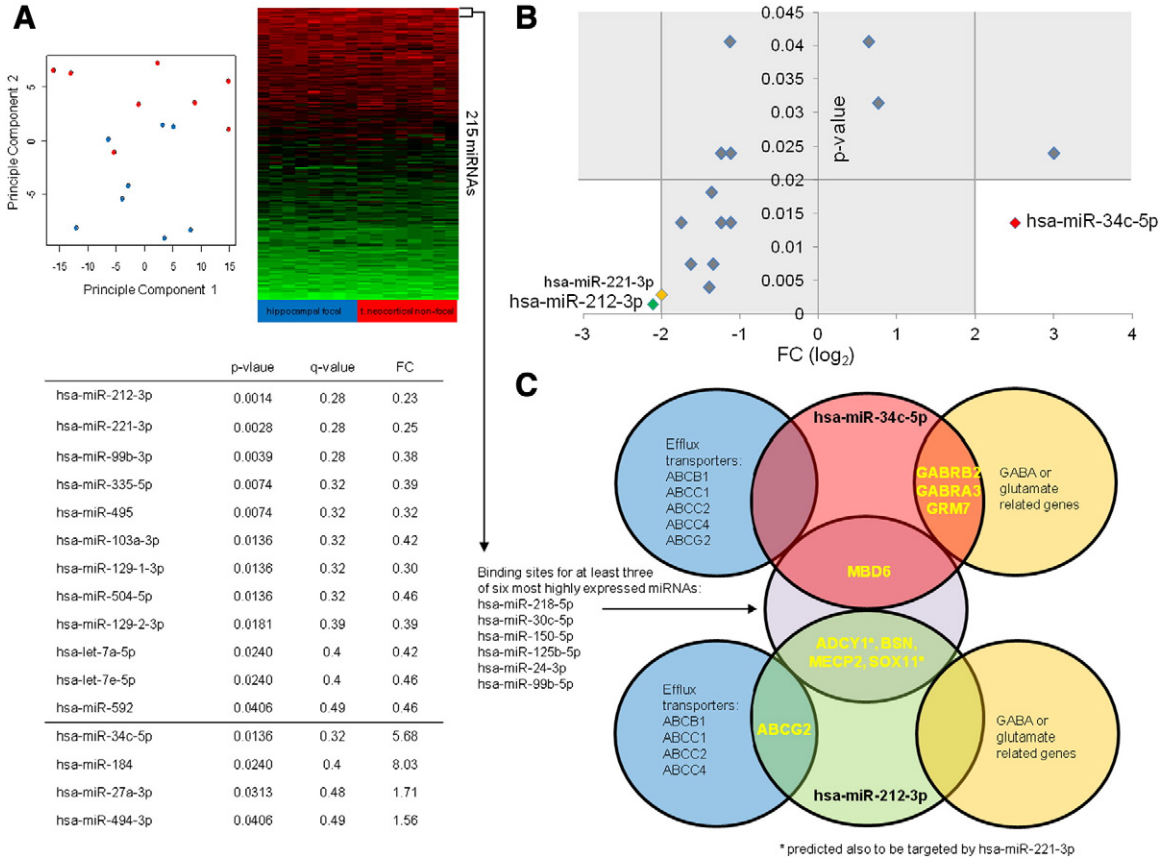
The second approach was hypothesis-generating. Genes were selected which were predicted by GOmir (Roubelakis et al., 2009) to be targeted by at least three of the six most highly expressed miRNAs (top 3% of all detected miRNAs in the examined brain samples) and additionally by one of the selected differentially expressed candidate miRNAs. The intention underlying this filtering approach was that highly expressed miRNAs in brain may play an important role in maintaining optimal expression of genes that are critical for normal neuronal function.

Cell culture

Human hepatoblastoma HepG2 cells (DMSZ, Braunschweig, Germany) were cultured in RPMI 1640 medium (GE Healthcare, PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS Superior, Biochrome, Berlin, Germany) and 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco by lifetechnologies, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 atmosphere.

Cloning of reporter gene plasmids and site directed mutagenesis

Reporter gene plasmids were generated to confirm miRNA/mRNA-3′-UTR interference between selected miRNAs and putative target genes. 3′-UTR sequences containing the predicted binding site for the respective candidate miRNA were amplified from human genomic DNA using forward primers containing a PmeI restriction site and reverse primers containing a XbaI (GABRA3: XhoI) restriction site (Table 2) and subsequently inserted into the pmirGLO vector (Promega, Madison, WI, USA). Table 2 shows the positions of the inserted 3′-UTR sequences and location of the predicted miRNA binding site. A total of 14 plasmids were generated for nine putative target genes since some of the 3′-UTR sequences were predicted to have more than one binding site for the respective candidate miRNA. Predicted target sequences for hsa-miR-212-3p were subsequently subcloned into the psiCHECK™2 vector (Promega), since co-transfection of pmiRGLO vector and pre-miR-212-3p showed strong off-target effects. To facilitate ligation of the inserts into the correct 5′- to 3′-direction downstream of the reporter sequence, the multiple cloning site of the psiCHECK™2 vector had to be slightly modified. For exact procedure see supplementary methods. The resulting plasmid DNA is from now on referred to as psicheck2S vector. T4 DNA ligase was purchased from Invitrogen by Life Technologies (Carlsbad, CA, USA) and Antarctic Phosphatase and restriction enzymes were purchased from NewEngland Biolabs (Frankfurt, Germany). Plasmid DNA was amplified in DH5α competent *E. coli* cells. The correctness of inserted target sequences was verified by capillary sequencing of



	p-vlaue	q-value	FC
hsa-miR-212-3p	0.0014	0.28	0.23
hsa-miR-221-3p	0.0028	0.28	0.25
hsa-miR-99b-3p	0.0039	0.28	0.38
hsa-miR-335-5p	0.0074	0.32	0.39
hsa-miR-495	0.0074	0.32	0.32
hsa-miR-103a-3p	0.0136	0.32	0.42
hsa-miR-129-1-3p	0.0136	0.32	0.30
hsa-miR-504-5p	0.0136	0.32	0.46
hsa-miR-129-2-3p	0.0181	0.39	0.39
hsa-let-7a-5p	0.0240	0.4	0.42
hsa-let-7e-5p	0.0240	0.4	0.46
hsa-miR-592	0.0406	0.49	0.46
hsa-miR-34c-5p	0.0136	0.32	5.68
hsa-miR-184	0.0240	0.4	8.03
hsa-miR-27a-3p	0.0313	0.48	1.71
hsa-miR-494-3p	0.0406	0.49	1.56

Fig. 1. Array-based miRNA screen and target gene filtering strategies. A) PCA: blue circles, hippocampal focal tissues; red circles, temporal neocortical non-focal tissues. Heatmap: 215 miRNAs detected in both tissue types were sorted in terms of increasing overall median expression values (red high, green low expression level). B) Volcano plot: Comparison of 215 miRNAs between 8 focal hippocampal and 8 non-focal temporal neocortical tissues with a Mann–Whitney *U* test and subsequent correction for multiple testing revealed sixteen miRNAs having an unadjusted p-value ≤0.05. For detailed information about p-values, FDR adjusted p-values (q-values) and mean fold changes (FC) see table to the left of Venn diagram. The most prominent candidates (hsa-miR-34c-5p, hsa–miR-212-3p and hsa-miR-221-3p) showed a p-value ≤0.02 and a $\log_2$ transformed fold change ≥2.0. C) Venn diagram showing the results of target gene filtering for hsa-miR-34c-5p and hsa–miR-212-3p. Both a hypothesis-guided and a hypothesis-generating approach were used as described in the Materials and Methods section.

plasmid DNA (GATC Biotech, Konstanz, Germany), using the respective forward or reverse primers shown in Table 2.

To confirm the exact locations of predicted hsa-miR-212-3p and hsa-miR-132-3p miRNA binding sites in SOX11-3′-UTR, mutations were introduced into the target sequence of the SOX11-V2 plasmid by site-directed mutagenesis and appropriate mutagenesis primers; the distal wild type sequence ACCTTTTTT**TGA**ATT**GACTGTT**T (predicted by TargetScan 5.2 and miRanda) and the proximal wild type sequence A**GGCA**G**TG**CG**CTGGAG**GA**TG**A (predicted by RNAHybrid) were changed into ACCTTTTTT***TCT***ATT***CTA*****TCCT**T and A***GAC***A**GGG**CG***TACAGG***GA**TG**A, respectively (nucleotides of seed sequence are in bold, mutated nucleotides are italicized). A plasmid carrying both mutated sequences was also generated. Each mutagenesis PCR reaction was performed by using 5 ng of wildtype SOX11-V2 plasmid DNA as template and the KAPAHiFi™ PCR Kit (PeqLab, Biotechnologie GmbH, Erlangen, Germany) according to standard recommendations of the manufacturer. Dpn-I (New England Biolabs) restriction digestion was immediately conducted for 1 hour at 37 °C. The resulting mutant plasmids were named SOX11-V2-mut-TS, SOX11-V2-mut-RHy and SOX11-V2-mut-dko. Mutated target sequences were verified by capillary sequencing of plasmid DNA (IKMB, Kiel, Germany), using the respective forward (FW-SOX11-V2) or reverse primers (RV-SOX11-V2) as shown in Table 2. All described primers and oligonucleotides were purchased from Sigma-Aldrich (Munich, Germany).

Reporter gene assays with mutated SOX11-V2 plasmids, co-transfected with pre-miR-212-3p, Pre-miR-132-3p or pre-miR-NC, at a concentration of 2.5 nM each, were performed as described below.

Transient transfection with precursor-miRNAs and reporter gene assays

The interaction between predicted target mRNA-3′-UTR sequences and selected candidate miRNAs was evaluated in HepG2 cells, chosen for their putative non-brain like endogenous miRNA expression profile. Cells were reverse transfected in 96-well plates by covering 20 µl of transfection complex with 80 µl of cell suspension (120,000 cells/ml) per well. For generation of transfection complexes Opti-MEM I medium (Gibco), siPORT™ NeoFX™ transfection reagent (Ambion), reporter gene plasmid DNA (45 ng/well) and precursor miRNA (pre-miR) molecules of candidate miRNAs (pre-miR-34c-5p or pre-miR-212-3p) in a final concentration of 2.5 and 10 nM or 1.0 and 2.5 nM, respectively, were combined according to the manufacturer's recommendation. Endogenous cellular miRNA expression was normalized by co-transfecting each reporter plasmid with precursor miRNA negative control molecules (pre-miR-NC) at the same concentrations as described above. Potential indirect miRNA-mediated effects on promoter activity were assessed by co-transfecting empty pmirGLO or psicheck2S vectors with the same concentrations of pre-miRs of candidate miRNAs or pre-miR-NC. All pre-miR molecules were purchased from Ambion. Four hours after transfection, medium was replaced by fresh growth medium. The activities of both firefly and *Renilla* luciferases were determined 48 h after transfection using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's recommendation and Veritas Software coupled to the Veritas microplate luminometer (Tuner Biosystems, Sunnyvale, CA, USA). The *Firefly* luciferase

activities of pmirGLO plasmids were normalized to *Renilla* luciferase activities to control for transfection efficiency, and the *Renilla* luciferase activities of psicheck2S plasmids were normalized to *Firefly* luciferase activities, respectively. Seven biological replicates were included in each experiment, and each experiment was repeated with two different concentrations of pre-miR-molecules. Reporter gene activities in cells co-transfected with plasmids and pre-miR are shown relative to those obtained from cells co-transfected with the same plasmid and pre-miR-NC.

Isolation and culture of primary rat cortical neonatal neurons

Mixed cortical cultures containing both neurons and astrocytes were prepared as described previously (Brewer, 1995) and modified recently (Zhao et al., 2006). In brief, medium I (DMEM containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin) and medium II (neurobasal medium containing 2% B27 supplement, 0.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) (Gibco) were pre-warmed. Immediately after sacrifice the cerebral cortices of 0 to 1 day neonatal Wistar rats were dissected and dispersed using 0.25% trypsin (Gibco), followed by gentle trituration. After washing twice with neurobasal medium and once with medium I at 1000 rpm for 4 min, cells were counted and seeded in poly-L-lysine (0.1 mg/ml, Sigma-Aldrich) pre-coated 4-well culture plates in medium I. After 3 h incubation, culture medium I was replaced with culture medium II, and the cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

The time-dependent expression of Sox11 mRNA, miR-212-3p and miR-132-3p was monitored at 13 different time points (0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144 hours) with total RNA isolation using a mirVana miRNA Isolation Kit (Ambion).

Electroporation of primary rat cortical neonatal neurons with miRNA inhibitors (anti-miRs)

Cortical cells were isolated as described above. Five aliquots of 4.8 x 10^6 cells each were centrifuged for 5 min at 80 *g*. Cell pellets were resuspended in 100 μl Nucleofector® solution (Amaxa® Rat Neuron Nucleofector® Kit, Lonza, Cologne, Germany) and spiked with the miRNA inhibitor molecules anti-miR-212-3p, anti-miR-132-3p or anti-miR-NC at a concentration of 150 nM, or with both anti-miR-212-3p (150 nM) and anti-miR-132-3p (150 nM) or with anti-miR-NC (300 nM) (Ambion), respectively. After 10 minutes of incubation, electroporation was conducted using an Amaxa® Nucleofector® I (Lonza) and the appropriate electroporation program (G13) according to the manufacturer's recommendation. Cell suspension from each electroporation was diluted and aliquoted in two poly-L-lysine- (0.1 mg/ml) pre-coated 4-well culture plates, containing 500 μl Medium I per well. Medium I was replaced with medium II after 3 h incubation and cells were maintained as described above. At eight different time points (0, 12, 24, 48, 60, 72, 96 and 144 hours) total RNA was isolated for determination of Sox11 mRNA expression.

Quantification of time-dependent mRNA and miRNA expression in rat cortical neurons

Total RNA isolated from naïve or transfected primary rat cortical neonatal neurons was subjected to DNAse treatment (TURBO DNA-free™ Kit, Ambion). Next, 100 ng of total RNA per sample was reverse transcribed (Transcriptor High Fidelity cDNA Synthesis Kit, Roche, Mannheim, Germany) and cDNA quantification was performed using TaqMan® assays (rat Sox11: assay ID: Rn00584322_s1, rat ACTB (β-Actin): Cat. No.: 4352340E) and TaqMan® Universal Master Mix II, no UNG. In parallel, total RNA (50 ng per sample) was reverse transcribed using a pool of miR-212-3p, miR-132-3p and U6snRNA specific RT primers and the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). After an additional pre-amplification step similar to that described above, miRNA expression was determined with individual TaqMan® MicroRNA assays (assay ID: 000515, 000457 and 001973, respectively) and TaqMan® Universal Master Mix II, no UNG (all assays and master mixes were purchased from Applied Biosystems). All quantitative real-time PCR reactions were run on an ABI prism 7900HT (Applied Biosystems) in duplicate. qRTPCR data were expressed as $2^{-\Delta Ct}$, using rat ACTB or U6snRNA as endogenous controls.

Staining and immunofluorescence microscopy of transfected rat neurons

Primary neuronal cells were transfected by electroporation with pmaxGFP™ vector (Lonza) and additionally with identical anti-miRs or anti-miR combinations (anti-miR-212-3p, anti-miR-132-3p, anti-miR-NC) in identical concentrations as described above. Cells were cultured on poly-L-lysine (0.1 mg/ml) pre-coated cover slips placed in 4-well culture plates. Cells were fixed after four different time points (24, 48, 72, and 96 hours after plating) with 4% paraformaldehyde in PBS for 1 h at 4 °C, washed, and permeabilized with 0.3% TritonX-100 in PBS for 5 min. After blocking with 1% BSA, primary neuronal cells were incubated with the primary rabbit anti-rat Sox11 antibody (H-290) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:300) at 4 °C overnight. After washing with PBST (PBS containing 0.1% Triton X-100), the cells were incubated with the corresponding secondary antibody (Alexa Fluor®546-conjugated goat anti-rabbit antibody; Molecular Probes by Life Technologies, Leiden, The Netherlands) in 0.5% BSA at 37 °C for 1 h in the dark. After washing twice with PBST and once with PBS and subsequent drying at room temperature, the cells were mounted in ProLong® Gold Antifade Reagent (Molecular Probes). For qualitative analysis, stained primary neuronal cells were analyzed with a Leica DMR fluorescence microscope (Leica, Wetzlar, Germany).

In silico analysis of putative transcription factor binding sites

The region 3000 bp 5′-upstream of the ATG start of rat and human SOX11 (Ensembl gene browser ID: ENSRNOG00000030034, ENSG00000176887) as well as 3000 bp 5′-upstream of miR-212/132 (ENSRNOG00000035476, ENSG00000267195) were evaluated for putative transcription factor binding sites using the Transfac® database (release 2013.2) (Matys et al., 2006). For each transcription factor binding site search the software tool was preset to "vertebrate_non_redundant_minFP" and "profile-specific-cutoff".

Statistical analysis

Comparisons of miRNA expression between the human brain tissue types were performed as described above. GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California, USA) was used to perform a Mann–Whitney *U* test for differences in normalized reporter gene activities between HepG2 cells co-transfected with respective candidate pre-miRs and either empty vector or one of the various cloned 3′-UTR target sequence reporter gene plasmids. Linear regression was performed to test for positive or negative correlation between mRNA and miRNA expression in primary rat neurons over the monitored time period.

Results

miRNA screen in human brain tissue

Out of 754 miRNAs on a TaqMan® low density array, 215 miRNAs were detected in all 16 (8 hippocampal focal, 8 temporal neocortical non-focal) human brain tissue samples. miRNAs with the highest median overall expression levels (top 3% of all detected miRNAs) were hsa-miR-125b-5p, hsa-miR-218-5p, hsa-miR-150-5p, hsa-miR-30c-5p, hsa-miR-24-3p, and hsa-miR-99b-5p. These miRNAs were not differentially

Table 2
Vector systems and inserted target gene 3′-UTR sequences used in reporter gene assays for target gene evaluation of hsa-miR-212-3p and hsa-miR-34c-5p.

Target gene	mRNA Acc. number	Position of 3′-UTR sequence of insert	miRNA predicted to bind to 3′-UTR sequence	Location of miRNA binding site in 3′-UTR sequence	Name and sequence of forward primer	Name and sequence of reverse primer	Name of vector system	Name of vector construct
ABCG2	NM_004827.2	484-663	hsa-miR-212-3p	588-607[†]	FW-ABCG2-V1: 5‘-CTCGTTT‘AAACAAAAA AAGCCACCGTGATAGAAA	RV-ABCG2-V1: 5′-GAGT*CTAGAGTTCCA GAAATGGTGCAAGAAT	psicheck2S	ABCG2-V1
ABCG2	NM_004827.2	1328-1462	hsa-miR-212-3p	1411-1417[‡]	FW-ABCG2-V2: 5‘-CTCGTTT‘AAACCTCTTT GCTCAGCTTCCCTTAAT	RV-ABCG2-V2: 5′-GAGT*CTAGATTAGTG GAAAAGTGGTGAAATCG	psicheck2S	ABCG2-V2
ADCY1	NM_021116.2	1-612	hsa-miR-212-3p	268-290[†]	FW-ADCY1-V1 5‘-CTCGTTT‘AAACTGGAG CCCACGTGGGCCTCTGGGGTGC ACA	RV-ADCY1-V1 5′-GAGT*CTAGATTCTCC CCAGACGGTCCAGTTCA AGAGTGGAC	psicheck2S	ADCY-V1
ADCY1	NM_021116.2	2853-3622	hsa-miR-212-3p	3246-3252[‡]	FW-ADCY1-V2 5‘-CTCGTTT‘AAACGATTC GAAGTGTCACAGAGATGGTGCC GTC	RV-ADCY1-V2 5′-GAGT*CTAGAAGCATG GCCAAGCAAGGGCTGAGGTG GGTGTG	psicheck2S	ADCY-V2
SOX11	NM_003108.3	51-523	hsa-miR-212-3p	509-515[‡]	FW-SOX11-V1: 5‘-CTCGTTT‘AAACTTGG GAGGAAGTTGTAGTGG	RV-SOX11-V1: 5′-GAGT*CTAGACCCTAG AGTAACAGTTGTGC	psicheck2S	SOX11-V1
SOX11	NM_003108.3	6500-7199	hsa-miR-212-3p	6620-6641[‡,§], 6954-6973[†]	FW-SOX11-V2: 5‘-CTCGTTT‘AAACCTCCC TTGAATCACATAGGG	RV-SOX11-V2: 5′-GAGT*CTAGAGAAGCT GGTTAGATCGAAGC	psicheck2S	SOX11-V2
BSN	NM_003458.3	362-616	hsa-miR-212-3p	485-505[†]	FW-BSN V1: 5‘-CTCGTTT‘AAACTACTT CCCACAGCCGCCTTTT	RV-BSN V1: 5′-GAGT*CTAGAGGCCA CATGGAGGTCTGCT	psicheck2S	BSN-V1
BSN	NM_003458.3	1240-1537	hsa-miR-212-3p	1382-1392[‡]	FW-BSN V2: 5‘-CTCGTTT‘AAACGGGGA ATGTGGCACTGTCT	RV-BSN V2 5′-GAGT*CTAGA TGCCA CAGACTGAGAATTTCAC	psicheck2S	BSN-V2
MECP2	NM_004992.3	6294-7203	hsa-miR-212-3p	6591-6610[†], 6887-6893[‡,&]	FW-MECP2: 5‘-CTCGTTT‘AAACTCCAG AAACTTGTTCTTCCAAAGCAG	RV-MECP2: 5′-GAGT*CTAGATACTTTA AACATAGTTACAATACAGA	psicheck2S	MECP2
GABRA3	NM_000808.3	1-1066	hsa-miR-34c-5p	221-227[‡,§], 256-282[†], 722-728[‡,§]	FW-GABRA3: 5‘-CTCGTTT‘AAACATAGT GGTGGCAGTGCAGCAAC CAGAGCACTGT	RV-GABRA3: 5′-GAGC[#]TCGAGTGCCTG GGCCAGGGTGTGGAATGTGG AGTGTA	pmiRGLO	GABRA3
GABBR2	NM_005458.7	259-425	hsa-miR-34c-5p	393-418[†]	FW-GABBR2 V1: 5‘-CTCGTTT‘AAACAAACA AGGAGGCGCTGGGATAT	RV-GABBR2 V1: 5′-GAGT*CTAGATGTGCA GAGGCAGTTTCTTCAGA	pmiRGLO	GABBR2-V1
GABBR2	NM_005458.7	2282-2433	hsa-miR-34c-5p	2384-2389[‡]	FW-GABBR2 V2: 5‘-CTCGTTT‘AAACTCTTTT ATATGCAGCAAACACACC	RV-GABBR2 V2: 5′-GAGT*CTAGACATGCA GTAGGGGTACGAGAAATT	pmiRGLO	GABBR2-V2
GRM7	NM_181874.2	1-1196	hsa-miR-34c-5p	281-287[‡,§], 266-287[†]	FW-GRM7: 5‘-CTCGTTT‘AAACCTTTT GACTGCTTTCCCAAAGGCCCTG CTGCA	RV-GRM7: 5′-GAGT*CTAGAAAAAAG TTAACCAAAGTATTTTATTGAT ACAT	pmiRGLO	GRM7
MBD6	NM_052897	365-869	hsa-miR-34c-5p	663-673[‡,§], 756-789[†]	FW-MBD6: 5‘-CTCGTTT‘AAAC CCCTTCT TAAAGCCAACAGGC	RV-MBD6: 5′-GAGT*CTAGAATAGAA AAGCAGTGGAGGCCC	pmiRGLO	MBD6

‘Pme I restriction site.
Predicted by target gene prediction database [†]RNAHybrid, [‡]Targetscan 5.2, [§]miRanda, [&]PicTar.
* Xba I restriction site.
[#] Xho I restriction site.

expressed between focal and non-focal tissue. PCA of the expression of the 215 miRNAs revealed similarity in the expression profiles of miRNAs in samples from hippocampal focal compared to temporal neocortical non-focal brain tissue samples (Fig. 1A). A comparison of miRNA expression levels in focal and non-focal tissue identified 16 miRNAs showing an unadjusted p-value ≤0.05: hsa-miR-212-3p, hsa-miR-221-3p, hsa-miR-99b-3p, hsa-miR-335-5p, hsa-miR-495, hsa-miR-103a-3p, hsa-miR-129-1-3p, hsa-miR-504-5p, hsa-miR-129-2-3p, hsa-let-7a-5p, hsa-let-7e-5p, hsa-miR-592 were down-regulated and hsa-miR-34c-5p, hsa-miR-184, hsa-miR-27a-3p and hsa-miR-494-3p were upregulated in focal compared to non-focal tissue. Of these 16 miRNAs, three showed a p-value ≤0.02 and in parallel a $\log_2$-transformed fold change of ≥2.0 (Fig. 1B). hsa-miR-34c-5p was upregulated 5.7 fold in hippocampal focal tissues compared to non-focal temporal neocortical tissues ($p = 0.014$, q-value = 0.32), whereas hsa-miR-212-3p and hsa-miR-221-3p were down-regulated 76.9% ($p = 0.0014$, q-value = 0.28) and 74.9% ($p = 0.0028$, q-value = 0.28), respectively. These data were even more pronounced, when miRNA expression of hippocampal focal and temporal neocortical non-focal tissue of additional five therapy-resistant MTLE patients were regarded (see supplementary Fig. 1S). Since demographic and histhopathological information was not available for those patients, these data were not included in the initial comparison.

Nevertheless, since the initial array data revealed relatively high FDR adjusted p-values (q-values), the differential expression between hippocampal focal and temporal neocortical brain tissue for hsa-miR-34c-5p, hsa-miR-212-3p and hsa-miR-221-3p was re-evaluated with individual TaqMan assays. The differential expression of hsa-miR-34c-5p and hsa-miR-212-3p was confirmed (mean change: 8.3-fold increase ($p = 0.008$) and 33% decrease ($p = 0.04$), respectively). In contrast, no significant difference in expression of hsa-miR-221-3p between hippocampal focal and temporal neocortical non-focal brain tissue was detected. Based on these findings, hsa-miR-34c-5p and hsa-miR-212-3p were chosen for further functional studies.

In specimens of individuals without neurological disease obtained from autopsies no significant difference in expression of hsa-miR-212-3p between hippocampal and frontal cortical brain tissue was observed (Fig. 5B).

In silico search for target genes of candidate miRNAs

Putative target genes of hsa-miR-34c-5p and hsa-miR-212-3p were identified using two approaches. The aim of the first (hypothesis-guided) approach was to take into consideration common hypotheses about the pathophysiology of epilepsy syndromes, especially those showing an intractable phenotype such as MTLE. By using four different prediction databases (TargetScan 5.2, RNAHybrid, PicTar and miRanda), common ABC transporters (ABCB1, ABCC1, ABCC2, ABCC4 and ABCG2) often implicated in pharmacoresistance were tested for putative interaction with hsa-miR-34c-5p and hsa-miR-212-3p. In addition, two gene lists containing 564 (hsa-miR-34c-5p) and 341 (hsa-miR-212-3p) target genes were generated using the GOmir software application (Roubelakis et al., 2009) and the same databases. Given the critical role of gamma-aminobutyric acid (GABA) and glutamate as determinants of neuronal network excitability, these lists were selectively searched for genes involved in GABAergic and glutamatergic neuronal signal transmission. The efflux transporter ABCG2 was identified as a putative target of hsa-miR-212-3p and GABA B receptor 2 (GABBR2), glutamate receptor, metabotropic 7 (GRM7) and GABA A receptor, alpha 3 (GABRA3) were predicted targets of hsa-miR-34c-5p (Fig. 1C).

The second approach was designed to be hypothesis-generating. GOmir (Roubelakis et al., 2009) identified the following number of putative targets of the six highest expressed miRNAs (top 3% of miRNAs detected in all 16 investigated human brain samples): hsa-miR-218-5p ($n = 784$ predicted target genes), hsa-miR-30c-5p ($n = 1250$), hsa-miR-150-5p ($n = 214$), hsa-miR-125b-5p ($n = 670$), hsa-miR-24-3p ($n = 546$), hsa-miR-99b-5p ($n = 41$). These six lists were filtered for genes that were targeted by at least three of the six most highly expressed miRNAs and additionally by one of the selected differentially expressed candidate miRNAs. There is evidence from literature, that some of these miRNAs, namely hsa-miR-218-5p and hsa-miR-125b-5p, are typically brain-enriched miRNAs (Sempere et al., 2004) and McKiernan et al. also found hsa-miR-218-5p, hsa-miR-30c-5p, hsa-miR-125b-5p and hsa-miR-24-3p amongst the top 40 expressed miRNAs in healthy hippocampus (McKiernan et al., 2012a), supporting the validity of our array data. The intention underlying this filtering approach was to use the most highly expressed miRNAs to uncover genes that are more likely to be critical for normal brain function and to result in crucial functional impairments as a result of altered miRNA expression. The conduction of this approach revealed adenylate cyclase 1 (brain) (ADCY1), bassoon presynaptic cytomatrix protein (BSN), methyl CpG binding protein 2 (MECP2) and SRY (sex determining region Y)-box 11 (SOX11) to be targets of hsa-miR-212-3p, whereas ADCY1 was additionally predicted to be targeted by hsa-miR-218-5p, hsa-miR-125b-5p and hsa-miR-99b-5p; BSN by hsa-miR-218-5p, hsa-miR-30c-5p, hsa-miR-150-5p, hsa-miR-125b-5p and hsa-miR-24-3p; MECP2 by hsa-miR-218-5p, hsa-miR-30c-5p and hsa-miR-24-3p and SOX11 by hsa-miR-218-5p, hsa-miR-30c-5p, hsa-miR-125b-5p and hsa-miR-24-3p. Methyl-CpG binding domain protein 6 (MBD6) was predicted as a putative target of hsa-miR-34c-5p, and the 3′-UTR of MBD6 harbored additional putative binding sites for hsa-miR-30c-5p, hsa-miR-150-5p, miR-24-3p and hsa-miR-99b-5p (Fig. 1C).

Reporter gene assay after transient co-transfection of HepG2 cells with 3′-UTR target sequence plasmids and precursor-miRNAs

RNA interference between predicted 3′-UTR sequences of putative target gene mRNAs and selected respective candidate miRNAs was evaluated using reporter gene assays. The 3′-UTR sequence of GABRA3 mRNA was identified as a target of hsa-miR-34c-5p based on a reduction in normalized reporter gene activities of 39-46% relative to empty pmiRGLO vector following co-transfection with 10 nM ($p = 0.002$) and 2.5 nM ($p = 0.002$) pre-miR-34c-5p. The 3′-UTR sequences of GRM7 and of GABBR2 containing the predicted distal hsa-miR-34c-5p binding site were targeted after co-transfection of 10 nM pre-miR-34c-5p; a mean reduction in reporter gene activities of 37.5% ($p = 0.001$) and 32.4% ($p = 0.002$), respectively, was observed. The putative proximal binding site in the GABBR2-3′-UTR and the binding site in the 3′-UTR sequence of MBD6 were not targeted by hsa-miR-34c-5p (Fig. 2A and B).

Reporter gene activities of MECP2 and SOX11-V2 (containing 3′-UTR sequence with two more distal predicted hsa-212-3p binding sites) plasmids were significantly reduced by 1.0 nM (MECP2: 32%, $p = 0.03$; SOX11-V2: 30%, $p = 0.03$) and 2.5 nM (MECP2: 51%, $p = 0.003$; SOX11-V2: 64%, $p = 0.0007$) pre-miR-212-3p. Only the higher concentration of pre-miR-212-3p reduced luciferase activity of the ABCG2-V2 or ADCY1-V2 plasmids (21%, $p = 0.04$ and 36%, $p = 0.009$), respectively. Plasmids containing 3′-UTR sequences of BSN (BSN-V1 carrying a predicted proximal and BSN-V2 carrying a predicted distal binding site), and 3′-UTR sequences of ADCY1, SOX11 and ABCG2 with predicted proximal hsa-miR-212-3p binding sites showed no significant down regulation in reporter gene assays (Fig. 2 C and D).

Identification of binding sites of tandem miRNAs hsa-miR-212-3p and hsa-miR-132-3p in SOX11-3′-UTR

Reporter gene assays found the strongest interference between miRNA and its mRNA target sequence for hsa-miR-212-3p and SOX11. However, SOX11 was not previously verified as a target of hsa-miR-212-3p. Since it is known that hsa-miR-212-3p and hsa-miR-132-3p are not only processed from the same primary transcript, but also share a similar mature sequence and an identical seed region (tandem miRNAs) (Wanet et al., 2012), we hypothesized that SOX11 is not only targeted by hsa-miR-212-3p, but also by hsa-132-3p. The array analysis indicated a non-significant mean difference in hsa-miR-132-3p between hippocampal focal versus temporal neocortical non-focal human brain tissue (35% decrease, $p = 0.32$). Reporter gene assays were used to investigate further the miRNA/mRNA interaction and the location of binding sites of hsa-miR-212-3p and hsa-miR-132-3p in the SOX11-3′-UTR. The SOX11-V2 plasmid contained two different putative miRNA binding sites and was mutated at either the proximal or distal site or at both hsa-miR-212-3p binding sites (Fig. 3). Empty psicheck2S vector or wildtype or mutated SOX11-V2 reporter plasmids were co-transfected with pre-miR-212-3p or pre-miR-132-3p or pre-miR negative control in HepG2 cells at a concentration of 2.5 nM each. Treatment with pre-miR-212-3p resulted in a decrease in wild type SOX11-V2 reporter activity (55.6% decrease, $p = 0.002$); the activity of the single and double mutant plasmids was reduced to a lesser degree (SOX11-V2-mut-TS: 28.0% decrease, $p = 0.026$; SOX11-V2-mut-RHy: 43.9% decrease, $p = 0.004$; and SOX11-V2-mut-dko: 22% decrease, $p = 0.097$) (Fig. 3A). Similar results were found for treatment with pre-miR-132-3p; reporter gene activities were decreased 34.9% ($p = 0.026$), 22.2% ($p = 0.165$), 19.6 % ($p = 0.165$) and 12.6% ($p = 0.383$) for the wild type, SOX11-V2-mut-TS, SOX-11-V2-mut-RHy and SOX11-V2-mut-dko plasmids, respectively. These results identified two nucleotide sites (position 6620–6641 and 6954–6973 of SOX11-3′-UTR) that annealed to hsa-miR-212-3p and hsa-miR-132-3p, with the latter having a smaller influence on SOX11 expression compared to hsa-miR-212-3p. Moreover, since the mutation of the putative proximal binding site (SOX11-V2-mut-TS) led to less pronounced down-regulation of the reporter activity compared to mutation of the distal binding site (SOX11-V2-mut-RHy), hsa-miR-212-3p seems to have a stronger negative effect on SOX11 expression via the proximal than the distal binding site. Additionally, these results suggest that both binding sites work additively in controlling SOX11 expression via interaction with hsa-miR-212-3p as well as hsa-miR-132-3p.

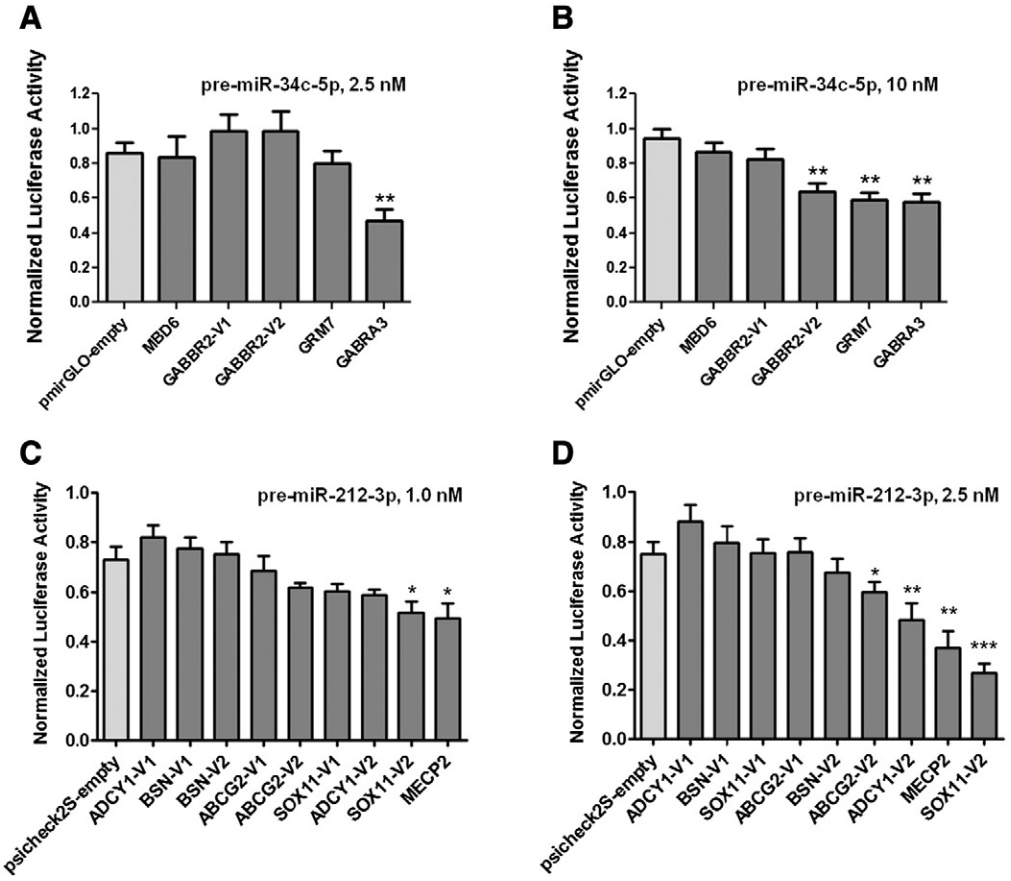


Fig. 2. Evaluation of 3′-UTR functional activity and regulation by miRNAs. Reporter gene activities were measured in HepG2 cells co-transfected with 3′-UTR target sequence plasmids and two concentrations of pre-miRs for candidate miRNAs hsa-miR-34c-5p (A and B) and hsa-miR-212-3p (C and D) predicted to interfere with the 3′-UTR sequence of target gene mRNAs ($n = 7$). To exclude potential effects of endogenous expressed miRNAs, activities have been normalized to activities obtained from cells co-transfected with respective 3′-UTR target sequence plasmids and pre-miR-negative control. V1-labeled plasmids contain respective 3′-UTR sequences containing predicted proximally located miRNA binding site(s) and V2-labeled plasmids contain respective 3′-UTR sequences containing predicted distally located miRNA binding site(s) (see also Table 2). Values are means ± standard error of the mean (SEM). Interference of candidate miRNAs with target mRNA were tested with a Mann–Whitney *U* test, whereas in each experiment normalized activities of cells co-transfected with empty vector plasmids and respective identical concentrations of pre-miR molecules were used as calibrator. *, **, *** indicate p-values ≤ 0.05, 0.01, and 0.001, respectively.

Quantification of time-dependent mRNA and miRNA expression in naïve and anti-miR-transfected neonatal rat neurons

Adult neurogenesis is an essential and widely conserved process in the majority of mammals. During neurogenesis, the neuronal transcription factor SOX11 is involved in initiation of neuronal differentiation. Protein sequences of human (acc. no.: NP_003099.1) and rat (acc. no.: NP_445801.1) SOX11 are highly homologous. Comparison of both sequences using the NCBI HomoloGene tool (http://www.ncbi.nlm.nih.gov/homologene?LinkName=protein_homologene&from_uid=4507161) revealed several human-rat sequence alignments with the highest percent identity of 96% found for the sequence alignment from position 1–139. The percentage of human sequence covered by alignment to the rat sequence was 82%. Thus, we hypothesized that rat Sox11 was similarly regulated by miR-212-3p/132-3p as its human homolog. An *in silico* search using RNAhybrid software identified a nucleotide sequence (position 354–374 of rat Sox11-3′-UTR, acc. no.: NM_053349.1) predicted to be targeted through hsa-miR-212-3p and hsa-miR-132-3p.

miR-212-3p/132-3p-mediated control of SOX11 expression was further investigated by examining the time-dependent expression of both miRNAs and rat Sox11 mRNA in differentiating neurons cultured in normal growth medium after isolation from neonatal rat brains. A significant inverse correlation was observed between Sox11 mRNA and both miR-212-3p ($R^2 = 0.33$, $p = 0.04$) and miR-132-3p ($R^2 = 0.34$, $p = 0.04$) over 144 hours in culture. However, closer inspection indicated that Sox11 mRNA and both miR-212-3p ($R^2 = 0.96$, $p = 0.0004$) and miR-132-3p ($R^2 = 0.61$, $p = 0.005$) were concurrently up-regulated over the initial 48 hr culture period. In contrast, from 60 to 144 hours Sox11 mRNA tended to show an inverse relationship with miR-212-3p ($R^2 = 0.45$, $p = 0.07$) and miR-132-3p ($R^2 = 0.28$, $p = 0.17$) expression, and Sox11 mRNA was suppressed compared to early time points (Fig. 4A and B). This observation is consistent with an initial common transcriptional mechanism controlling expression of Sox11 and the primary transcript containing the tandem miRNAs miR-212-3p and miR-132-3p, which subsequently act as negative post-transcriptional regulators of SOX11.

Quantification of time-dependent Sox11 mRNA expression and immunofluorescence microscopy of Sox11 protein in neonatal rat neurons transfected with anti-miRs

Time-dependent Sox11 mRNA expression was measured after transfection of anti-miR molecules antagonizing endogenous expression of miR-212-3p or miR-132-3p in freshly isolated neurons of neonatal rats. Antagonism of only one or both miRNAs resulted in a time-dependent down-regulation of Sox11 mRNA, which was more pronounced with anti-miR-132-3p than with anti-miR-212-3p. Interestingly, in neurons transfected with both anti-miR-212-3p and anti-miR-132-3p, Sox11 mRNA was not down-regulated and showed a tendency to be up-regulated over a 144 hr observation period (Fig. 5).

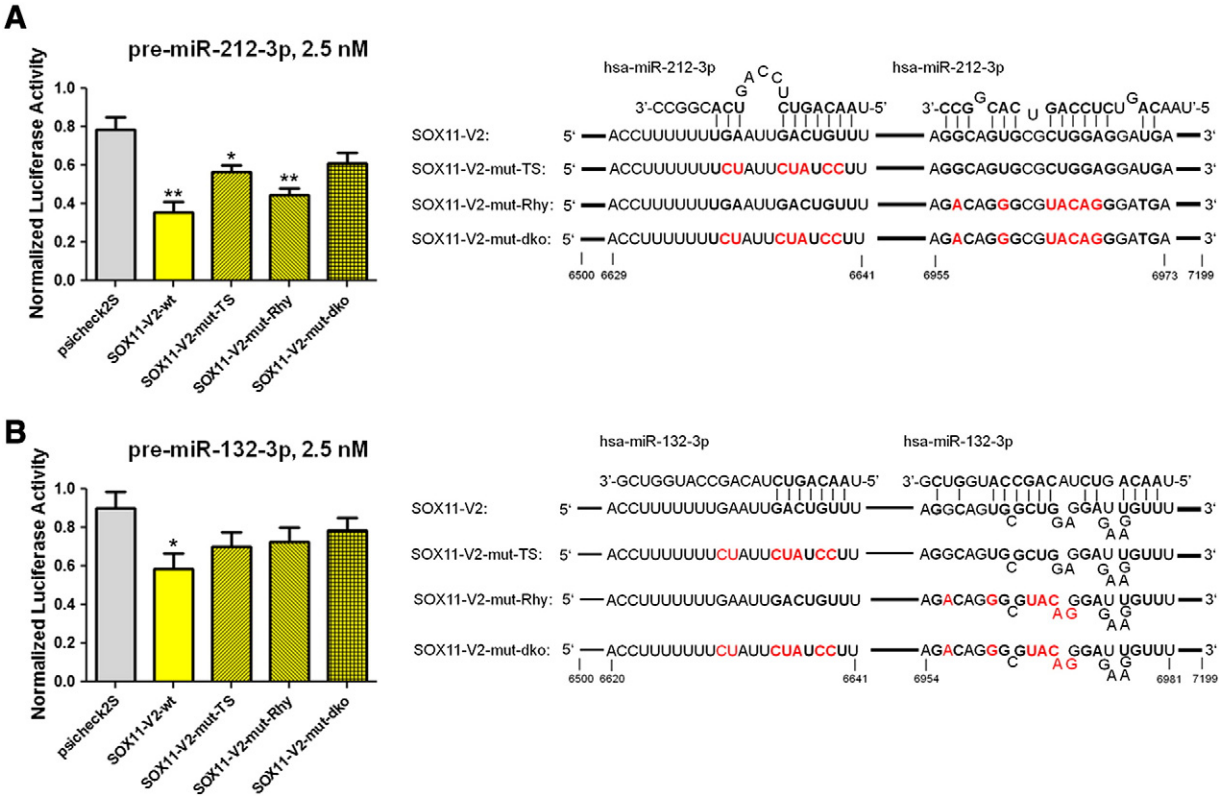


Fig. 3. Functional miRNA binding sites on SOX11-3′-UTR. Normalized reporter gene activities in HepG2 cells co-transfected with wild type or mutated SOX11-V2-3′-UTR target sequence plasmids and 2.5 nM of pre-miR-212-3p (A) or pre-miR-132-3p (B) ($n = 7$). All activities (mean ± SEM) have been normalized to activities obtained from cells co-transfected with respective 3′-UTR target sequence plasmids and pre-miR-negative control. * and ** indicate p-values ≤ 0.05 and ≤ 0.01, respectively. Bold characters indicate sequence complementary to miRNA seed sequence, red characters indicate mutated nucleotides. SOX11-V2-mut-TS contains mutated miRNA binding site predicted by Targetscan 5.2, SOX11-V2-mut-RHy contains mutated miRNA binding site predicted by RNAHybrid, and SOX11-V2-mut-dko contains SOX11-V2-3′-UTR sequence with both predicted binding sites mutated.

Immunofluorescence staining and microscopy in neurons 96 hours after transfection with anti-miR molecules revealed lower Sox11 protein in cells transfected with one anti-miR compared to cells transfected with anti-miR-212-3p and anti-miR-132-3p, with the lowest signal intensity seen in anti-miR-132-3p transfected neurons. Cells double transfected with anti-miR-212-3p and anti-miR-132-3p not only showed the strongest Sox11 protein signal, but also a less differentiated morphology with a reduced dendrite-like structure (Fig. 5).

Putative transcription factor binding sites in promoter region of rat and human SOX11 and primary transcript of miR-212-3p/132-3p

Based on the observed concurrent initial transcription of Sox11 and miR-212-3p as well as miR-132-3p in primary cortical rat neurons (Fig. 4), the question arose whether SOX11 is a transcription factor for its own regulator miR-212-3p/132-3p or rather SOX11 and the primary transcript of miR-212-3p/132-3p share a common set of yet unknown transcription factors.

Assuming a similar interplay between SOX11 and miR-212-3p/132-3p in man and rat, the 5′-UTR of rat and human SOX11 and the primary transcript for miR-212-3p/132-3p were scanned from position −1 to −3000 for putative transcription factor binding sites using the Transfac® database. Response elements for SP1 (primary accession ID: M00933), DR3 (primary accession ID: M00966) and EBOX (primary accession ID: M01034) were predicted for all four 5′-UTR sequences. All binding sites were classified as family matrix transcription factor binding sites, meaning that a group of related transcription factors may bind at those sites. Additionally, in all four sequences a factor specific binding site for PAX3 (primary accession ID: M00327) was found. Since no SOX11 binding site was identified in the 5′-UTR region of the primary transcript miR-212-3p/132-3p, the observed initial concurrent regulation of both transcripts may be mediated by shared transcription factors rather than via direct induction of miR-212/132 through SOX11.

Discussion

Neurogenesis persists in the subventricular zone (SVZ) of the temporal ventricles and the subgranular zone (SGZ) of the hippocampus throughout adulthood. In the SGZ, learning and physical exercise can stimulate neurogenesis, whereas neurotransmitters, growth factors, cytokines, and hormones stage-specifically influence proliferation of neural stem cell derived progenitor cells and their differentiation into mature neurons (Masiulis et al., 2011; Ming and Song, 2011). Moreover, in certain epilepsy types, e.g. MTLE as well as in animal epilepsy models, acute seizures can promote hippocampal neurogenesis (Parent et al., 1997, 2006; Thom, 2004). It is speculated that seizure-induced newborn granule cells exhibit altered molecular and physiological properties likely associated with altered excitability. In the chronic stage of epilepsy, however, in the hippocampus the number of neuronal stem cells (NSCs) seems not to change significantly. However, the process of proper differentiation of NSC progenies into mature neurons appears to be impaired (Crespel et al., 2005; Hattiangady and Shetty, 2008; Kralic et al., 2005; Kuruba et al., 2009). miRNAs are important regulators of cellular processes and are also involved in the fine-tuning of the stage-specific control of neurogenesis (Sun et al., 2012). Comparing two brain tissue types, one without the potential of adult neurogenesis (temporal neocortex) and one with signs of abnormalities in neurogenesis, the current study was designed to further explore the roles of miRNAs in aberrant epilepsy-related hippocampal cellular remodeling. A small set of 16 miRNAs were found to be differentially expressed between hippocampal focal and temporal neocortical non-

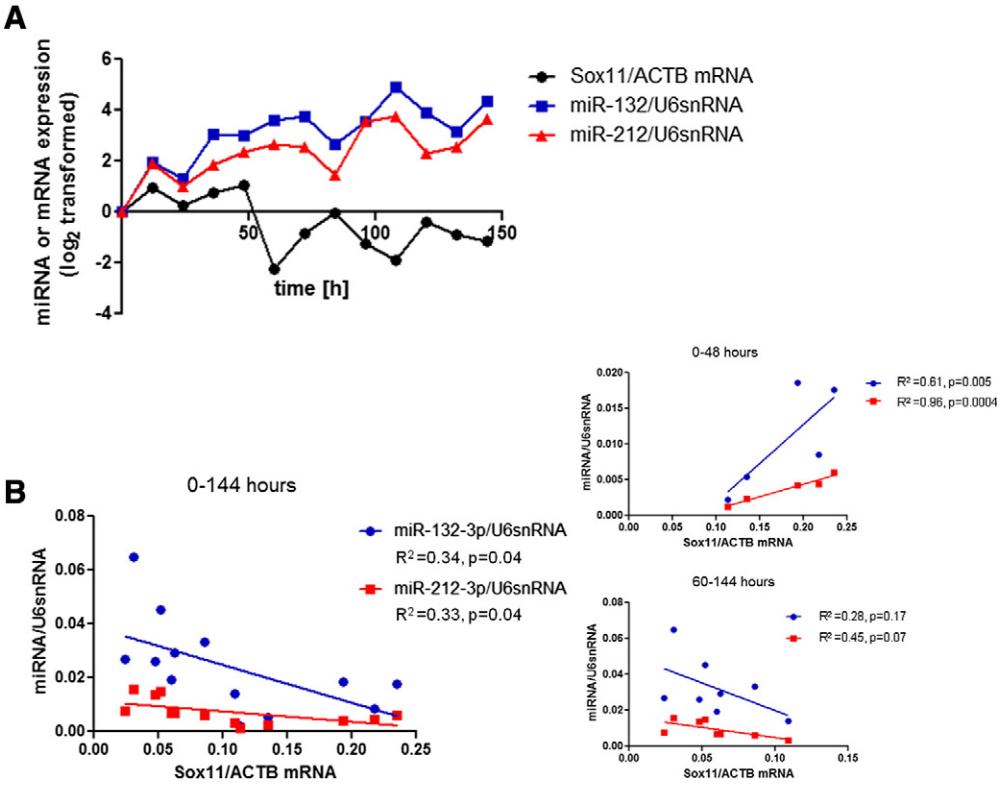


Fig. 4. Time-dependent changes in the expression of Sox11, miR-132-3p and miR-212-3p in differentiating rat neurons. (A) Time-dependent Sox11 mRNA and miR-132-3p and miR-212-3p expression in differentiating neonatal rat neurons. B) Linear regression of Sox11 mRNA and miR-132-3p and miR-212-3p expression over 144 hours (left panel) or from 0–60 hr and 60–144 hr (right panel).

focal brain tissue (Fig. 1B). Interestingly, the expression of hsa-miRNA-184 was elevated 8.0-fold in focal hippocampal tissue compared to non-focal neocortical tissue. hsa-miRNA-184 was previously shown to be up-regulated in mouse hippocampus eight hours after seizure preconditioning with an intraperitoneal injection of kainate (McKiernan et al., 2012b) and it was suggested that miR-184 protects against neuronal cell death (McKiernan et al., 2012b) and promotes proliferation of adult hippocampal neural progenitor cells, inhibiting their differentiation (Liu et al., 2010; Sun et al., 2012). Hippocampal miR-27a was also reported to be up-regulated (Hu et al., 2012) and let-7e was increased 24 hours and decreased 50 days after lithium-pilocarpine-induced status epilepticus (SE) compared to control rats (Song et al., 2011). hsa-miR-212-3p was reported to be differentially regulated in a time-dependent manner after electrically stimulated SE in rat hippocampus (Bot et al., 2013) and hsa-miR-221 was down-regulated in human hippocampi of TLE patients exhibiting hippocampal sclerosis (HS) (Kan et al., 2012). In the current study, hsa-miR-34c-5p was increased more than 5-fold in focal hippocampal tissue compared to temporal neocortical non-focal tissue. Although hsa-miR-34c-5p has not yet been associated with any epilepsy model, it shares an identical seed region with miR-34a, a miRNA that was up-regulated in the hippocampus 24 hours after lithium-pilocarpine-induced SE in rat (Hu et al., 2011) and after intra-amygdala-injection of kainic acid in mice (Sano et al., 2012). These data and our results imply that a certain set of miRNAs show an epilepsy-associated altered expression profile. However, the expression of some miRNAs varies as a function of the stage of disease and probably also with the epilepsy model.

Filtering for target genes of hsa-miR-34c-5p and hsa-miR-212-3p revealed a set of nine genes putatively involved in GABAergic and glutamatergic signal transmission (GABRA3, GABBR2, GRM7, BSN), neuronal cAMP formation (ADCY1), neuronal gene regulation (SOX11, MECP2, MBD6) and drug efflux (ABCG2). All except for BSN and MBD6 interacted with the miRNAs *in vitro*. A negative regulation of GRM7 3′-UTR and the distal GABBR2 3′-UTR by hsa-miR-34c-5p was detected (Fig. 2B). In addition to being an important excitatory neurotransmitter, glutamate is also necessary for the normal survival and integration of newborn granule neurons in the dentate gyrus, while also inhibiting proliferation of neuronal stem cells (Berg et al., 2013; Ming and Song, 2011; Tashiro et al., 2006). Glutamate mediates inhibitory effects on presynaptic calcium channels and on adenylate cyclase via the metabotropic glutamate receptor 7 (GRM7), a presynaptic expressed G-protein coupled receptor, thus controlling intracellular cAMP formation and glutamate release from the presynaptic axonal terminal (Benarroch, 2008). GRM7 was shown to be inversely expressed with miR-34a-5p in rat hippocampi after lithium and valproate treatment, suggesting an anticonvulsive mechanism of action of both drugs via miRNA-mediated gene regulation (Zhou et al., 2009). The current findings support the assumption of an overlap of target genes of miR-34a-5p and miR-34c-5p. $GABA_B$ receptor, also a G-protein coupled receptor, comprises subunits 1 and 2, and both subunits have to correctly dimerize to properly mediate inhibitory effects of GABA on voltage dependent calcium channels and activating effects on potassium channels, thus playing an important role in controlling neuronal excitability and hippocampal long term potentiation (Benarroch, 2012). Moreover, pharmacological inhibition of $GABA_B$ receptors led to increased proliferation of neuronal stem cells (Giachino et al., 2014).

GABRA3 3′-UTR showed the strongest negative interaction with miR-34c-5p (Fig. 2A and B). GABA is the most important inhibitory neurotransmitter in the nervous system, leading to hyperpolarization of neuronal membranes through $GABA_A$ receptors, which are chloride channels comprising a pentameric subunit protein structure. In SGZ, GABA is also involved in the control of the number of actively proliferating neural stem cells (NSCs) during neurogenesis via GABAergic interneurons (Masiulis et al., 2011). Interestingly, $GABA_A$ receptor subunit 3 as well as GABBR2 were found to be expressed at lower levels in leukocytes of patients with migraines compared to healthy controls

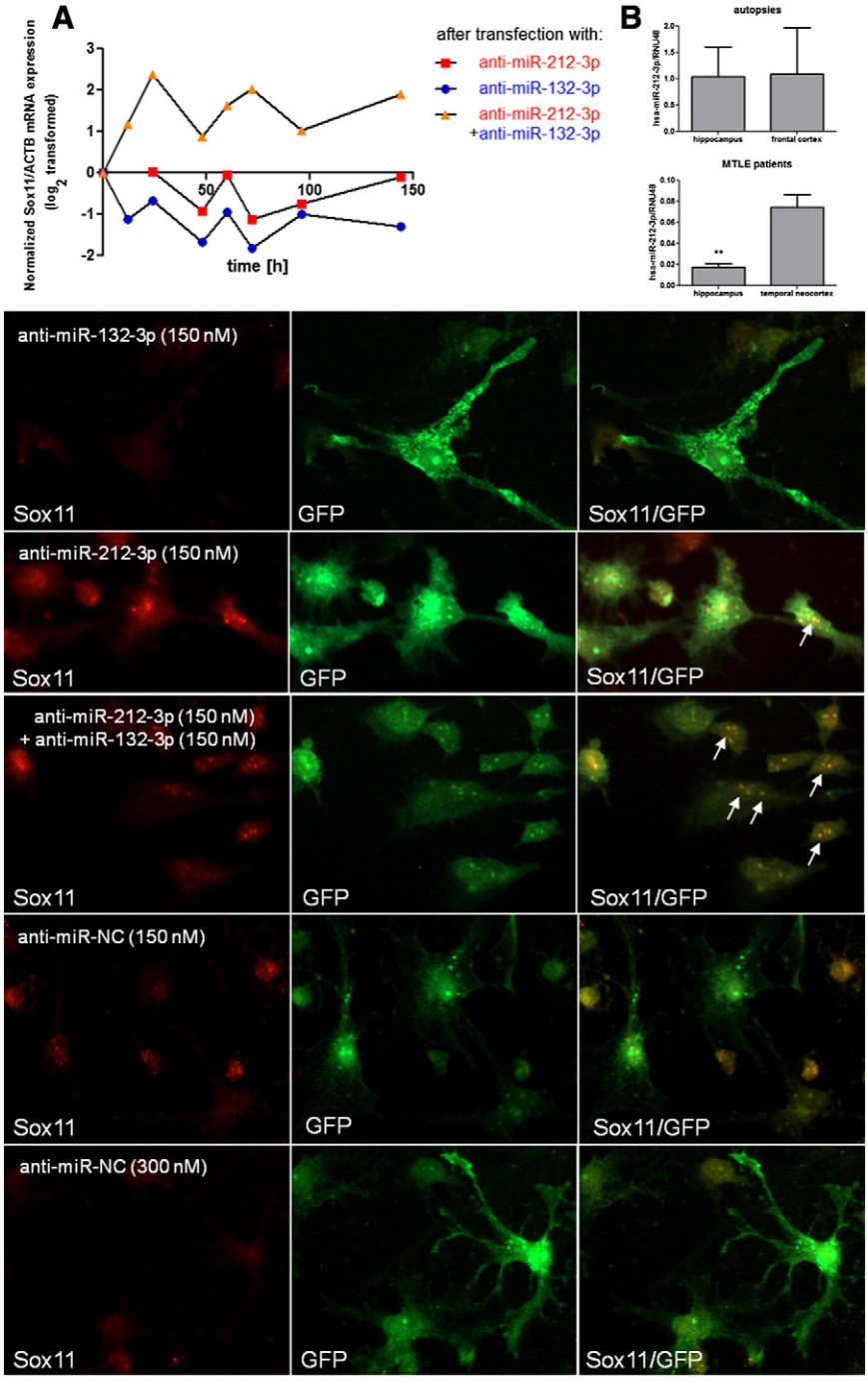


Fig. 5. A: Time-dependent Sox11 mRNA expression after transfection of neonatal rat neurons with anti-miR-132-3p or anti-miR-212-3p. Neonatal rat neurons were transfected with each anti-miR (150 nM) alone and in combination and expression is normalized to neurons transfected with anti-miR-negative controls in respective concentrations (upper panel). Immunofluorescence microscopy images 96 hours after transfection of neonatal rat neurons with anti-miR-132 (150 nM) or anti-miR-212 (150 nM) or anti-miR-132 and anti-miR-212 (each 150 nM), anti-miR-negative control (150 nM) or anti-miR-negative control (300 nM) are shown. The green fluorescent protein (GFP) expression vector pmaxGFP™ was used as a transfection control. Merged pictures show Sox11 protein (yellow signal) in anti-miR-transfected neurons. Arrows indicate intracellular Sox11 protein reservoirs (magnification: 400×). B: hsa-miR-212-3p expression (mean ± SEM) in hippocampal and frontal cortical brain tissue from autopsies of individuals without neurological disease (n = 8) (upper panel) and in hippocampal and temporal neocortical brain tissue of MTLE patients (n = 8) (array data). ** indicates p-values ≤ 0.01.

(Plummer et al., 2011), and a polymorphism in GABRA3 was associated with altered pharmacological treatment response of epilepsy patients (Hung et al., 2013).

ADCY1 has not previously been identified as a target of hsa-miR-212-3p (Fig. 2D). ADCY1 is mainly expressed in brain and plays a major role in neuronal cAMP formation. Via NMDA-receptor binding, calcium-influx and calmodulin activation, glutamate postsynaptically stimulates adenylate cyclase, thereby increasing intracellular cAMP concentration and inducing expression of various CREB (cAMP response element-binding protein) dependent genes (Carlezon et al., 2005). Those genes are involved in synaptic plasticity and in neurogenesis, dendritic growth and arborization, a necessary requirement for correct integration of newborn neurons (Nakagawa et al., 2002). However, after abnormal stimulation of NMDA receptors through glutamate, ADCY1 was reported to play a crucial role in neuronal excitotoxicity (Wang et al., 2007). Taken together, down-regulation of the Gi protein coupled receptors GRM7 and GABBR2 and a putative elevated expression of ADCY1 caused by the constellation of an increased expression

of hsa-miR-34c-5p and a decreased expression of hsa-miR-212-3p in chronic epileptogenic brain tissue may lead to abnormal increases in neuronal cAMP formation and neurogenesis, resulting in altered plasticity and hyperexcitable neurons. This scenario could additionally be strengthened by an altered functionality of $GABA_A$ receptors caused by a decreased availability of subunit 3, which was identified as a target of hsa-miR-34c-5p.

In this study we confirm that MECP2, a methyl CpG binding protein responsible for epigenetic control of neuronal gene expression, is a target of hsa-miR-212-3p (Im et al., 2010; Wada et al., 2010). Mutations (Rett syndrome) and gene duplication (MECP2 duplication syndrome) lead to severe impairment of neuronal development often accompanied with epileptic seizures (Diaz de Leon-Guerrero et al., 2011; Na et al., 2013; Ramocki et al., 2010). In adult neurogenesis, MECP2 was shown to control the miR-137/Ezh2 axis involved in neural stem cell differentiation (Szulwach et al., 2010).

In neurogenesis several neuronal transcription factors are sequentially expressed and control stage-specific protein expression patterns during proliferation, maturation and differentiation of newborn neurons (Faigle and Song, 2013; Hodge et al., 2012). One of these transcription factors is SOX11. The SOX protein family is classified in groups (A-H), with SOX11 belonging to the SOX-C group (Kamachi and Kondoh, 2013). To be functional, SOX proteins have to homo- or heterodimerize with partner proteins, for example with transcription factors belonging to the Pit-Oct-Unc (POU) or pairerd box (PAX) protein families (Kamachi and Kondoh, 2013). There is evidence that members of the SOX-B1 group (e.g. SOX2) initiate the expression of SOX-C group members during neuronal fate commitment of precursor cells and neuronal development (Bergsland et al., 2011). Moreover, it was shown that the expression of SOX11 is strictly confined to neuronally committed precursors and immature neurons, but is absent in mature neurons (Haslinger et al., 2009; Mu et al., 2012).

In our investigation, SOX11 3′-UTR sequence showed the most prominent interaction with hsa-miR-212-3p (Fig. 2C and D). Moreover, in the SOX11-3′-UTR two different miR-212-3p binding sites were identified. These sites were also targeted by hsa-miR-132-3p (Fig. 3). Both miRNAs are transcribed from the same primary transcript and exhibit identical seed sequences (Wanet et al., 2012). There was a clear inverse overall correlation between Sox11 mRNA and both miR-212-3p and miR-132-3p in neurons of neonatal rats. On closer inspection, however, over the first 48 hours an initial concurrent transcription of Sox11 mRNA and both miRNAs was observed. At 60 hours and later time points when miR-212-3p and miR-132-3p had reached a certain threshold, Sox11 was strongly down-regulated (Fig. 4). Antagonizing both miRNAs led to a clear attenuation of the time-dependent expression pattern of Sox11. Sox11 mRNA and protein were highest in neurons 96 hours after co-transfection with both anti-miR-212-3p and anti-miR-132-3p and these cells showed a less differentiated morphology compared to controls. Cells transfected with anti-miR-132-3p showed clear dendrite-like morphology, however, the dendrite structure was less branched compared to control cells (Fig. 5). These findings are supported by two recent reports describing the role of miR-132 in length, aborization, and integration of newborn neurons in the dentate gyrus (Luikart et al., 2011; Magill et al., 2010). It may be speculated that miR-132-3p is gradually more functionally relevant for arborization and branching of newborn neurons than for controlling Sox11 expression. In contrast, collective findings of these studies further suggest that miR-212-3p is predominantly involved in controlling Sox11 expression. It should be noted that the interference of miR-212-3p and miR-132-3p with the 3′-UTR of the rat Sox11 homolog has not been tested. However, an *in silico* analysis revealed a common binding site for both miRNAs in the 3′-UTR of rat Sox11.

Since no SOX11 binding site was found in the 5′-UTR of rat and human miR-212/132, the observed initial concurrent transcription of SOX11 and miR-212/132 could be controlled through common transcription factors. Candidate transcription factors include PAX3 and SP1; DR3 and E-Box (Enhancer box) sequences might also contribute to this shared regulation. As mentioned above, members of the SOX-B1 group (e.g. SOX2) can dimerize with proteins of POU and PAX families in order to initiate the transcription of their downstream target genes. The putative PAX3 binding site implies that not only members of the SOX-C family, but also miR-212/132 could be included in those target genes. The transcription factor binding site for SP1 and the E-box sequence (e.g. c-Myc binds to E-box) are involved in chromatin remodeling and transcription initiation; DR3 response elements can bind PXR (pregnane X receptor) /RXR (retinoid X receptor) or CAR (constitutive androstane receptor)/RXR heterodimers (Handschin and Meyer, 2003). Since several anticonvulsants (e.g. carbamazepine, phenobarbital and phenytoin) are PXR/CAR ligands (Handschin and Meyer, 2003), an interesting question for future studies is whether these anticonvulsants have a modifying effect on seizure-induced neurogenesis.

ABCG2 was identified as a weak target of miR-212-3p. Interaction between miRNA-212-3p and ABCG2 was also demonstrated in an earlier study (Turrini et al., 2012). ABCG2 is an important efflux transporter implicated in chemo-resistance of tumor stem cells (Mo and Zhang, 2012). Since it is also expressed at the blood brain barrier (Mo and Zhang, 2012), ABCG2 may influence the distribution of anticonvulsants in the central nervous system. Moreover, ABCG2 is also a stem cell factor used to separate stem cells from their more differentiated progeny cells (Mo and Zhang, 2012; Scharenberg et al., 2002). In the context of neurogenesis, a miR-212-3p mediated parallel control of SOX11 and ABCG2 may be involved in the maturation process of neuronal stem cells into mature granule neurons through sequential expression of distinct protein markers.

Several limitations of the study have to be addressed. By using whole hippocampal tissue sections, the possibility that the observed changes in miRNA expression may also originate from or affect other cell populations (e.g. astrocytes, oligodendrocytes) than granule cells cannot be completely excluded. It is also possible that some of the observed epilepsy-induced altered miRNAs are part of a repair process as reported earlier for miRNA-184 (McKiernan et al., 2012b). A reactive down-regulation of miR-212-3p with an up-regulation of SOX11 may indicate an attempt of forced neurogenesis, however, potentially accompanied by impaired neuronal differentiation or even increased production of astrocytes due to altered neuronal fate decision (Crespel et al., 2005; Hattiangady and Shetty, 2008; Kralic et al., 2005; Kuruba et al., 2009). Another critical point is the choice of control tissue. By comparing hippocampus and temporal neocortex it is difficult to distinguish between natural tissue-specific differences in miRNA expression and epilepsy-mediated differences. Recent investigations of Ziats and Rennert, however, uncovered a relatively small set of 30 miRNAs differentially expressed between cortical (prefrontal cortex) and hippocampal tissue in healthy adolescents (Ziats and Rennert, 2014). None of the dysregulated miRNAs observed in our study was amongst these candidates, whereas some of the identified miRNAs, as mentioned above, show disease-dependent alterations in other epilepsy-models (Bot et al., 2013; Hu et al., 2012; Kan et al., 2012; McKiernan et al., 2012b; Song et al., 2011). Moreover, we did not observe a significant difference in hsa-miR-212-3p expression between hippocampal and frontal cortical tissue from autopsies of individuals without neurological disease (Fig. 5B), additionally supporting the idea, that at least some of the identified miRNAs (e.g. hsa-miR-212-3p) may indeed be disease-mediated rather than tissue-dependently dysregualted. Although only a small overlap in putative disease-mediated dysregualted miRNAs were seen compared to other studies using normal hippocampus from autopsies as control tissue (Kan et al., 2012; McKiernan et al., 2012a), notable similarities were seen in the most highly expressed miRNAs in normal hippocampus compared to temporal neocortex and hippocampus of MTLE patients examined in this study (McKiernan et al., 2012a). Besides the bias of tissue-specific differences in miRNA expression, one advantage comparing hippocampal focal and temporal neocortical non-focal tissue within a patient (as mostly done in this study) may be the exclusion of

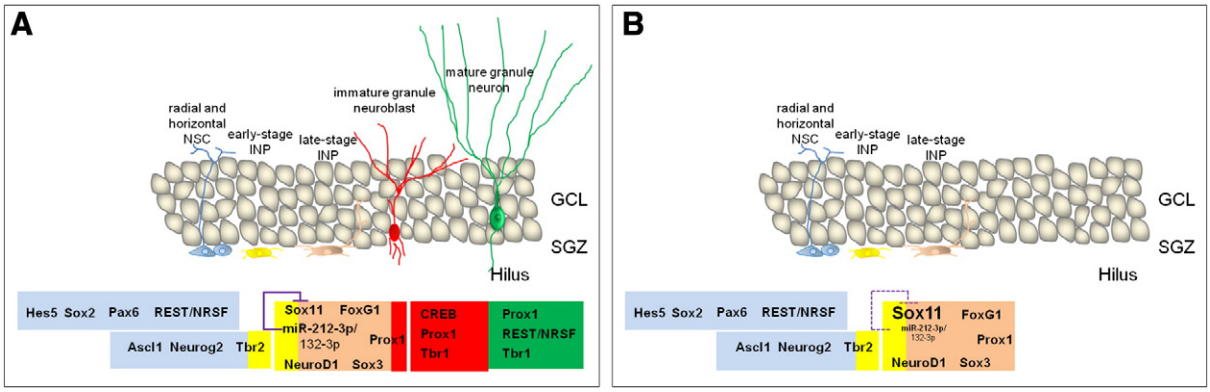


Fig. 6. Proposed mechanism for how dysregulated miR-212-3p/132-3p expression could affect the time- and stage-specific expression of neuronal transcription factors during differentiation and maturation of newborn granule neurons in SGZ. (A) miR-212-3p/132-3p controls the stage-specific expression of Sox11 during neuronal maturation. (B) Deregulated miR-212-3p/132-3p expression in MTLE may lead to uncontrolled Sox11 expression and may impair further differentiation of newborn neurons into mature granule neurons. Figure was modified from (Bischofberger, 2007; Hodge et al., 2012). NCS: neural stem cells, INP: intermediate neuronal progenitors, GCL: granule cell layer, SGZ: subgranular zone.

drug-induced alterations in miRNA expression, an important issue, since several anticonvulsant drugs exhibit marked effects on gene expression. Future prospectively planned studies in larger cohorts involving collection of both temporal neocortical and hippocampal tissue of normal brain is required to completely address these limitations.

Given that miR-212-3p is down-regulated in premature granule cells in the chronic stage of MTLE, the resulting increase in SOX11 expression would halt the process of neurogenesis at the stage of immature neurons and would impair the final maturation of neurons into mature granule cells, supporting the suggestion of an impaired neuronal differentiation in the chronic phase of epilepsy (Hattiangady and Shetty, 2008; Kuruba et al., 2009) (Fig. 6). Such a scenario could contribute to impaired integration of newborn neurons into existing hippocampal neural networks and altered excitability.

In conclusion, several target genes involved in neuronal signal transmission, neuronal gene regulation, neuronal cAMP formation and drug efflux were identified that may be altered through an epilepsy-mediated differential hippocampal miRNA expression pattern. Future studies should investigate whether these genes might serve as useful targets to improve pharmacological therapy options for the treatment of MTLE.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2015.02.025.

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